Estimation of L-Malate and Fumarate by Malic Decarboxylase of *Lactobacillus arabinosus*

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The manometric estimation of L-malate by the malic decarboxylase of *Lactobacillus arabinosus* 17-5, has been described by Korkes & Ochoa (1948), Ochoa, Salles & Ortiz (1950) and Blanchard, Korkes, del Campillo & Ochoa (1950). As both the bacterial suspensions and the acetone powder extracts used by these workers have fumarase activity, the method measures the sum of malate and fumarate. In the present work, a modification is described whereby L-malate and fumarate are measured separately, and improvements are introduced which are based on the finding that in intact cells the activity of malic decarboxylase is optimal in the presence of glucose, manganous chloride and potassium chloride (Nossal, 1951*b*). Part of this work has been communicated to the Biochemical Society (Nossal, 1951*a*).

DEVELOPMENT OF THE METHOD

Standard conditions for the assay of malic decarboxylase activity, and estimation of L-malic acid in pure solution

The main compartment of conical Warburg vessels contained 3.6 ml. malate solution (equivalent to 40 μ mol. DL-compound) and 0.1 ml. buffer-co-factor solution (1 g. glucose, 1 g. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 14.9 g. KCl and 16.5 ml. 3*M*-sodium acetate buffer, pH 5.0, in 100 ml.). The side arm contained the bacterial suspension (0.2 ml.) prepared as previously described (Nossal, 1951*b*), and 0.1 ml. of buffer-co-factor solution. The gas space contained air. The bath temperature was 35°. These conditions had previously been found to be optimal for the quantitative decarboxylation of malate.

The rate of CO_2 evolution for 15 min. after mixing the contents of side arm and main compartment was proportional to the quantity of malic decarboxylase present, provided that the rate was not higher than 25 μ l./min. The activity of the bacteria is expressed as μ l. CO_2 /mg. dry wt./hr. (Q_{CO_2}), calculated from the maximum rate of CO_2 evolution (generally attained in the period 5–15 min. after mixing). For the estimation of L-malic acid the same conditions are used, up to 3.5 ml. of the sample to be analysed (brought to pH 5) being placed in the main compartment.

The effect of growth conditions on the yield of malic decarboxylase. The basal medium used in the following experiments was that described previously (Nossal, 1951*b*). The yield of enzyme increased with the malate concentration of the medium up to a level of 0.1 *M*-DL-malate, above which growth was inhibited. The Q_{CO_2} values of cells obtained

from media containing 4×10^{-3} , 2×10^{-2} and 0.1 *M*-DL-malate respectively were 205, 340 and 380, and the yields of cells (in mg. dry wt./100 ml. medium) were 61, 76 and 102 respectively.

The amount of enzyme produced after varying periods of incubation is shown in Table 1. It will be seen that production of the enzyme ceased earlier than growth. As the enzyme is an adaptive one, it was thought that the cessation of enzyme formation may have been due to the complete

Table 1. *Effect of growth period on yield of enzyme*

(1 ml. 20 hr. sub-culture inoculated into 500 ml. of medium (Nossal, 1951*b*). 100 ml. lots withdrawn at intervals, spun, washed twice, made up to 5.0 ml. with distilled water and stored at 2°. Activity of all samples estimated under the standard conditions after harvesting the last sample. Growth temperature 40°. $Q_{\text{CO}_2} = \mu$ l. CO_2 evolved/mg. dry wt. cells/hr.)

Time of withdrawal (hr.)	Yield of cells/100 ml. (mg. dry wt.)	Q_{CO_2}	Enzyme yield per 100 ml. medium (mg. dry wt. $\times Q_{\text{CO}_2}$)
10	5.7	480	2720
14	13.0	372	4830
18	14.8	320	4740
22	16.5	293	4840
34	16.0	296	4740

decomposition of the substrate. In fact, a second addition of 0.1 vol. 1 *M*-DL-malate after 12 hr. incubation increased the yield of enzyme at 24 hr. by about 30%. Incubation beyond 24 hr. did not increase the yield further. At 40° the yields of cells and enzyme were 10–20% lower than at 30°. Omission from the medium of acetate (which acts as a buffer) decreased the yield of enzyme by about 15%. In the standard procedure, therefore, the organism was grown at 30° for 24 hr. on the medium described previously, and after 12 hr. a second addition of 0.1 vol. sterile 1 *M*-DL-malate was made. The cells were washed with two lots of water (0.2 and 0.1 vol. of the medium respectively) and made up with 0.1 *M*-KCl to approximately 20% (wet wt.) suspensions.

The effect of enzyme concentration on the time required for complete decarboxylation. Under the standard conditions, 20 μ mol. L-malate were quantitatively decarboxylated in 50–90 min. by 2.5 mg. (dry wt.) fresh cells, in 20–30 min. by 7 mg. and in 15 min. by 14 mg. Routinely, 4 mg. were used, so that 500 ml. medium yielded enough cells for 150 determinations. The time required for the complete decarboxylation of 20 μ mol. L-malate by 4 mg. cells in the absence of inhibitory substances was about 45 min.

Specificity. Under the conditions described for the assay of decarboxylase activity (except that malate was replaced by other substances), 20 μ mol. of the following compounds

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did not yield measurable amounts of CO_2 in the time required for the complete decarboxylation of $40\text{ }\mu\text{mol}$. DL-malate: *dextro*-isocitrate, citrate, DL-lactate, L- α -hydroxy-glutarate, α -hydroxyisobutyrate, DL- β -hydroxybutyrate, DL-citramalate ($\text{CO}_2\text{H} \cdot \text{C}(\text{OH})(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$), hydroxymalonate, DL-tartrate, α -keto-glutarate, succinate, maleate, malonate, thiomalate, disaccharides, hexoses and pentoses.

The only substances which formed CO_2 were fumarate, oxaloacetate and pyruvate. Fumarate reacted twenty to fifty times more slowly than equivalent amounts of malate, owing to the presence of a weak fumarase in the bacteria. The rate of decarboxylation of oxaloacetate was of about the same order as that of malate. The rate of CO_2 evolution from pyruvate differed greatly with pyruvate concentration.

Table 2. *Loss of activity of cell suspensions on storage at 2°*

(Standard conditions for assay of decarboxylase activity; 2 mg. dry wt. of cells used throughout; in all cases the stored cells were pre-incubated with the buffer-co-factor solution for about 1 hr. before mixing with the malate. The percentage loss of activity was calculated by comparing the rates of the CO_2 evolution for the first 30 min. after mixing with that originally observed with fresh suspensions.)

Exp. no.	Suspension medium for storage	Percentage loss of activity				
		1st week	3rd week	5th week	7th week	9th week
1	Water	28	40	59	62	73
	0.5 M-KCl	11	34	35	32	43
	0.3 M-KCl	5	26	25	28	32
	0.1 M-KCl	0	18	20	20	25
2	Water	.	34	57	.	65
	0.1 M-KCl	.	0	8	.	15
	0.1 M-NaCl	.	12	18	.	20

Oxaloacetate can be removed by addition of 0.1 ml. of 0.4% aniline, which does not inhibit the decarboxylation of malate. Interference by fumarate and pyruvate is dealt with below.

Storage of the bacterial suspensions. Washed aqueous suspensions lose their malic decarboxylase activity fairly rapidly when stored at 2°; addition of glucose accelerates the inactivation, but KCl or NaCl greatly retard the loss of activity (Table 2). The activity can also be preserved in the deep freeze at -12°; freezing and thawing five times had little effect on the activity, especially when the cells were

suspended in 0.1 M-KCl. Routinely, cells suspended in 0.1 M-KCl were stored at 2°, for up to 6 weeks.

Reactivation of suspensions. Samples of bacterial suspensions which had lost part of their activity owing to prolonged storage in water could in general be partially reactivated by the addition of cozymase (Table 3), or by pre-incubation at 30° in 2.5×10^{-2} M-acetate buffer, pH 5.0, with 2.5×10^{-3} M-glucose and 10^{-4} M-nicotinic acid for 60 min. Both findings indicate that some of the loss of activity can be attributed to loss of cozymase, but even large excesses of cozymase never restored the original activity completely. Thus other factors are also responsible for the inactivation.

Inhibition. The following substances (brought to pH 5 with HCl or NaOH if necessary), at the final concentrations indicated did not affect the decarboxylation appreciably: cyanide (10^{-2} M), fluoride (10^{-2} M), azide (10^{-2} M), sulphide (10^{-2} M), NaCl (M), NH_4Cl (M), malonate (10^{-2} M), maleate (10^{-2} M), succinate (10^{-2} M), fluoroacetate (10^{-2} M), hydroxylamine (10^{-2} M), semicarbazide (10^{-2} M), 8-hydroxyquinoline (10^{-3} M), *p*-nitrophenol (10^{-2} M), trichlorophenol (10^{-3} M), BaCl_2 (10^{-2} M), ZnCl_2 (10^{-2} M), octyl alcohol (saturated) and 'Tween 20' (0.2%).

Tungstate, trichloroacetate, trioctylamine, 2,4-dinitrophenol, iodoacetate, *n*-hexadecyltrimethylammonium bromide (cetavlon), CuSO_4 , $\text{Al}_2(\text{SO}_4)_3$ and FeSO_4 were inhibitory at higher concentrations (Table 4). At lower inhibitor concentrations the inhibition was overcome by using greater amounts of cells except in the cases of cetavlon and trioctylamine.

Estimation of malate in the presence of fumarate. When equal quantities of fumarate and L-malate are present, e.g. $20\text{ }\mu\text{mol}$. (equivalent to $445\text{ }\mu\text{l}$. CO_2) of each, $10\text{--}20\text{ }\mu\text{l}$. CO_2 are formed from fumarate over the period required for the complete decarboxylation of malate. A correction may be made for the presence of fumarate by measuring the CO_2 output over twice the period required for the complete decarboxylation of malate, which gives recoveries to within 5% of the theoretical (Table 5).

Most of the fumarase activity of washed cells suspended in 0.1 M-KCl disappeared on storage for 2-4 weeks at 2°, whilst the malic decarboxylase activity was largely retained. With such aged suspensions, the following recoveries from $40\text{ }\mu\text{mol}$. DL-malate (equivalent to $445\text{ }\mu\text{l}$. CO_2) were obtained in the presence of $20\text{ }\mu\text{mol}$. fumarate, without applying correction factors (μl . CO_2): 442, 450, 446, 447, 443, 436, 452, 440, 445; mean: 444.

Table 3. *Reactivation of cell suspensions with cozymase*

(Standard conditions for assay of decarboxylase activity, but all cell samples pre-incubated in main compartment for about 1 hr. before mixing with malate.)

Exp. no.	Description of sample	Cells used (mg. dry wt.)	Period over which gas evolution was measured (min.)	Cozymase added* (mg.)	CO_2 evolution (μl .)
1	7 weeks old	2	30	.	219
		2	30	0.3	330
2	18 weeks old	4	90	.	232
		4	90	0.5	452
3	15 weeks old	2	90	.	(complete)
		2	90	.	52
		2	90	0.3	243

* The sample used was prepared from yeast and was 50% pure.

Table 4. *Inhibition of the malic decarboxylase*(Standard conditions for the estimation of malate; 40 μ mol. DL-malate, inhibitors brought to pH 5 if necessary.)

Inhibitor	Final concentration (M)	Time for complete decarboxylation of uninhibited control (min.)	Time for complete decarboxylation in presence of inhibitor (min.)	Notes
Sodium tungstate	2×10^{-2}	40	.	Half-decomposition attained in 60 min.
	2.5×10^{-3}	40	50	.
Trichloroacetate	10^{-2}	40	90	.
	10^{-3}	40	40	.
Triethylamine	1 drop/cup	45	.	Only 100 μ l. evolved in 45 min.
2:4-Dinitrophenol	10^{-2}	40	.	Almost total inhibition
	10^{-3}	40	120	.
	10^{-4}	40	60	.
Iodoacetate	2×10^{-2}	40	100	.
	2.5×10^{-3}	40	70	.
n-Hexadecyltrimethylammonium bromide (cetavlon)	10^{-2}	45	.	Almost total inhibition
	10^{-4}	30	.	Almost total inhibition
	10^{-4}	30	.	Almost total inhibition
CuSO ₄	10^{-2}	30	80	.
	10^{-3}	30	30	.
Al ₂ (SO ₄) ₃	10^{-2}	30	.	Incomplete after 60 min.
	10^{-3}	30	50	.
FeSO ₄	10^{-2}	30	60	.
	10^{-3}	30	40	.

An alternative procedure which eliminates the interference by fumarate is removal by chemical reactions. In pure solutions, excess saturated bromine water completely removed fumarate in 1 hr. at room temperature; this treatment, however, is unsatisfactory in biological material where it produces substances inhibitory to the malic de-

sample was then brought to pH 5 by the addition of approx. 0.2 ml. 10N-NaOH, using 0.1 ml. 0.04% bromocresol green as an internal indicator; water was added to give a final volume of 4.0 ml. After centrifugation, 3.0 ml. of the supernatant were used for the analysis. By this method, recoveries of malate from samples containing equal amounts of malate and fumarate were within 1-2% of the theoretical. The procedure proved satisfactory in biological materials.

Estimation of malate in the presence of pyruvate. If the sample to be assayed contains not more than 10 μ mol. pyruvate, the additional CO₂ output due to pyruvate is small and steady, and a similar correction to that applied above for fumarate may be used, again giving recoveries within 5%. In an experiment with 20 μ mol. DL-malate (equivalent to 223 μ l.) and 10 μ mol. pyruvate, the CO₂ evolution over the first 45 min. (during which complete decarboxylation was observed in a control without pyruvate) was 255 μ l. and in the next 45 min. 39 μ l. The malate recovery was thus 216 μ l. (97%). In a second experiment with 40 μ mol. DL-malate and up to 20 μ mol. pyruvate the recoveries were 451-459 μ l. (102-103%).

This correction cannot be applied to samples containing higher concentrations of pyruvate or small amounts of malate. In such cases, interference by pyruvate can be eliminated by addition of excess hydroxylamine or semicarbazide (Blanchard *et al.* 1950), for example, 10^{-2} M final concentration, neither of which appreciably affects the decarboxylation of malate. Recoveries to within 1% of the theoretical were obtained from samples containing 40 μ mol. DL-malate and 30 μ mol. pyruvate, by adding 35 μ mol. of either hydroxylamine or semicarbazide. In the standard procedure 0.2 ml. 0.1M-semicarbazide should be added to samples suspected to contain pyruvate.

Table 5. *Recovery of malate in the presence of fumarate*(Standard conditions for the estimation of malate; 40 μ mol. DL-malate; time required for complete decarboxylation of control: 40 min.)

Fumarate added (μ mol.)	CO ₂ evolution (μ l.)		Corrected value for malate	Percentage error
	1st 40 min.	2nd 40 min.		
.	450	.	450	1
2.5	453	1	454	2
5.0	469	8	461	3
10.0	474	11	463	4
20.0	486	25	461	3
40.0	513	50	463	4
80.0	548	89	459	3

carboxylase. More generally applicable is the reduction of fumarate to succinate by Zn and H₃PO₄ (Krebs, Smyth & Evans, 1940). To 3.0 ml. of the sample, in a 10 ml. centrifuge tube, were added 0.1 g. granulated Zn (20-30 mesh), 0.10 ml. 0.1M-CuSO₄, and 0.10 ml. 50% (w/v) H₃PO₄. After 45 min. at room temperature another 0.1 ml. portion of H₃PO₄ was added; reduction was complete within 90 min. and the

Estimation of fumaric acid

When fumarase and fumarate are added to the malic decarboxylase system, fumarate is converted quantitatively to lactate and CO_2 , the reaction proceeding to completion due to the continuous decarboxylation of the malate formed from fumarate. The fumarate content of a sample containing both malate and fumarate is given by the difference between an estimation done with added fumarase (which gives the sum of malate and fumarate) and a malate estimation on an aliquot in which fumarate has been removed by reduction or a malate estimation using stored fumarase-free cells.

Crude fumarase (from sheep heart) was rapidly inactivated by the decarboxylase system at pH 5.0 (see below), and a powerful fumarase preparation is therefore required. The following three yeast preparations proved suitable sources of fumarase: 1.0 ml. yeast maceration juice, as described by Krebs & Johnson (1937); 0.2–0.5 ml. of a 1:1 wet wt. aqueous suspension of baker's yeast frozen with solid CO_2 once and thawed at room temperature; 0.2 ml. of 1:1 wet wt. aqueous suspension of baker's yeast crushed in the cold without abrasives (Hughes, 1951). In all cases 0.2 ml. 0.2M-NaF should be added to reduce the blank CO_2 production of these preparations which were (under the conditions of the estimation): 30–60 μl . for 1.0 ml. yeast maceration juice; up to 50 μl . for 0.5 ml. frozen-thawed baker's yeast suspension; up to 30 μl . for 0.2 ml. crushed baker's yeast suspension (all in the presence of fluoride). These blank values can be reduced to about 10 μl . by 3 hr. dialysis against running tap water, which causes but little loss of fumarase activity. None of the materials retain their fumarase activity for more than a few days at 2° , but they remain active for several months when stored at -12° .

For the estimation of small amounts of fumarate, the most suitable material was found to be the cloudy supernatant of a centrifuged suspension of crushed baker's yeast which had been dialysed for 3 hr. against running tap water at 20° ; this material shows no blank gas output or uptake under the conditions of the estimation. Where the Hughes bacterial press is not available, dialysed suspensions of frozen-thawed baker's yeast are recommended.

Table 6 shows examples of estimations of malate and fumarate in separate solution; the cell suspension used had been stored in 0.1M-KCl at 2° for 3 weeks and the crushed baker's yeast at -12° for 4 weeks. When using frozen-thawed baker's yeast suspension, an O_2 uptake was occasionally observed in the control cups, in which case the estimation was repeated anaerobically.

Inactivation of fumarases by *Lb. arabinosus*. Suspensions of *Lb. arabinosus* rapidly inactivate fumarases from both animal tissues and yeasts on incubation at pH 5.0. Table 7 shows an experiment in which yeast fumarase was incubated for 1 hr. at 30° with and without the bacterial suspension and its activity assayed after that time. It can be seen that 60% of the fumarase activity was lost by pre-incubation with the bacteria. No loss of malic decarboxylase activity was observed in control cups containing malate as the substrate. In another experiment, 90% inactivation was observed with 2 ml. hog-heart fumarase preparation (1:4 wet weight aqueous homogenate) after 2 hr. pre-incubation with the decarboxylase system. The mechanism of this inactivation has not been studied. For the estimation of fumarate by the decarboxylase method, a considerable excess of concentrated fumarase is added so that the estimation goes to completion before inactivation begins to interfere with it.

Recoveries of malate and fumarate from biological materials. Recovery was satisfactory in all cases, suggesting that there are not normally interfering substances present in biological material; examples of recovery experiments are shown in Table 8, from which it can be seen that with one exception the estimations were within $\pm 2\%$ of the theoretical.

Preparation of samples for analysis. Deproteinization is not necessary, but it may be required to destroy enzymes in samples to be analysed. This can be done by addition of 0.1 vol. 2N-trichloroacetic acid and by placing this mixture in a boiling-water bath for 60 min. which destroys enzymes and at the same time removes trichloroacetic acid quantitatively. Care must be taken to note the change of volume of the sample during this treatment, and graduated test tubes were therefore used. Instead of trichloroacetic acid, 0.1 vol. 2N-HCl may be added,

Table 6. *Course of CO_2 evolution during estimations of malate and fumarate*

(Standard conditions, using 5 mg. dry wt. cells, suspended in 0.1M-KCl, and stored 3 weeks at 2° . The fumarase used was 0.2 ml. 1:1 wet wt. aqueous suspension of crushed baker's yeast, stored 4 weeks at -12° .)

Substrate (20 μM)	Fumarase added	Total CO_2 evolution (μl .)								
		10	20	30	40	50	60	70	80	90 min.
L-Malate	—	125	260	364	411	429	439	440	440	440
Fumarate	+	100	215	309	366	414	429	441	443	443
Fumarate	—	.	.	10	.	.	12	.	.	15

Table 7. *Inactivation of fumarase by pre-incubation with *Lactobacillus arabinosus**

(Standard conditions for the estimation of malate plus fumarate; 0.2 ml. 1:1 crushed baker's yeast suspension in the main compartment of all cups. Cell suspension in one side arm and substrate in the other.)

Cup	Substrate	Comments	CO_2 evolution in first 60 min. (μl .)
1	DL-Malate (40 μmol .)	Cells tipped into main compartment after equilibration, substrate 1 hr. later	{ 444 (complete) 163
2	Fumarate (20 μmol .)		
3	DL-Malate (40 μmol .)	Cells and substrate tipped into main compartment 1 hr. after equilibration	{ 442 (complete) 404
4	Fumarate (20 μmol .)		

Table 8. *Recoveries of malate and fumarate from biological materials*

(The materials were prepared by grinding the tissue or plant with equal volumes of water and M-trichloroacetic acid. After centrifugation, trichloroacetic acid was removed by immersion of the supernatant for 60 min. in a boiling-water bath. Standard conditions for the estimation of malate and malate + fumarate.)

Material	Added substrate	Fumarase added	Total CO ₂ * evolved (μl.)
Sheep heart	40 μmol. DL-Malate	—	446
Sheep heart	20 μmol. Fumarate	+	440
Sheep heart	40 μmol. DL-Malate, 20 μmol. fumarate; reduced	—	444
Sheep heart	40 μmol. DL-Malate, 20 μmol. fumarate; reduced	+	450
Sheep heart	20 μmol. DL-Malate; 10 μmol. fumarate	+	452
Sheep heart	20 μmol. Fumarate; reduced; then 20 μmol. fumarate added	+	441
Guinea pig kidney	20 μmol. Fumarate	+	432
Tomato	20 μmol. Fumarate	+	455
Pear	40 μmol. DL-Malate	—	442
Swede	40 μmol. DL-Malate	—	446
		Mean	445

* Corrected for the malate and fumarate content of the biological material; the sheep heart was a washed mince and contained no measurable amounts of malate or fumarate.

followed by 2 min. immersion in a boiling-water bath. In both cases the pH of the sample is adjusted to about 5 with NaOH or HCl after heating.

APPLICATIONS OF THE METHOD

The malate and fumarate content of biological materials. Malate and fumarate were estimated in all materials in the supernatants of samples ground with sand and trichloroacetic or hydrochloric acids. The supernatants contained the same concentration of malate as the uncentrifuged whole mixtures. Animal tissues generally contained small amounts of the two acids, and only the sum of malate and fumarate was estimated. The following values (in μmol./g. wet weight) were obtained: guinea pig: muscle, 3–5; liver, 2–3; kidneys, 1–3; whole brain, 1. Pigeon: liver, 5–13; breast muscle, 1–4; heart, 1–2. Pigeon liver gave the highest values, a result probably connected with the presence of the highly active carbon dioxide-fixation enzyme systems. After ten-fold concentration of extracts of sheep kidney and sheep liver by evaporation at 100° their malate and fumarate contents could be estimated: Sheep kidney contained 0.16 μmol. malate and 0.05 μmol. fumarate/g. wet weight and sheep liver 0.43 μmol. malate and 0.12 μmol. fumarate/g. wet weight. These give a ratio of malate to fumarate of about 3, which is approximately the expected value for tissues containing fumarase, and having a pH of 7.4. Marshall, Friedberg & Da Costa (1951) report malate concentrations of 0.36–3.43 mg./100 ml. (0.03–0.26 μmol./g.) in rat and mouse tissues.

Exceptionally high concentrations of malate were found in the haemolymph of *Gastrophilus intestinalis* larvae (collected during February), already known to contain relatively large concentrations of succinate (Levenbook, 1950). From 21 to 32 μmol. malate/ml. and from 3 to 6 μmol.

fumarate/ml. were found. These acids, therefore, make up a large portion of the anion deficit of the haemolymph described by Levenbook. This author observed great variations in the concentrations of glucose, lactate and ammonia in the haemolymph, depending on whether the larvae were collected and washed at 38 or 0°; such variations were not observed for malate and fumarate.

All specimens of plant materials contained malate, but the amounts varied (μmol./g. wet wt.): apple, 200; rhubarb, 120; cabbage leaf, 95; shallot, 90; pear, 75; lemon, 70 (per ml. of juice); carrot, 55; onion, 50; celery stalks, 35; tomato, 30; orange, 25 (per ml. of juice); cauliflower leaf, 20; potato, 18; grapes, 17.

The diurnal variation of malic acid concentration in Crassulacean leaves (see Bennett-Clark, 1933; Krebs & Eggleston, 1944) was confirmed. The malate concentration of *Bryophyllum calycinum* leaves after exposure to daylight was 10 (in May), 23 (in February) μmol./g. wet weight, rose to 60–90 μmol./g. wet weight after keeping the plant in the dark for 16 hr., and returned to its original level on re-exposure to light. The leaves of other succulent plants also contained high concentrations of malate: *Sedum praealtum*, 20–28 μmol./g. wet weight; *Crassula argentea*, 50 μmol./g. wet weight.

Determinations of malate plus fumarate showed that none of the above fruits or leaves contained measurable amounts of fumarate (i.e. more than 5% of the malate value), but leaves of *Myrrhis odorata* and of *Glaucium flavum* contained 13 and 99 μmol. fumarate (and 15 and 46 μmol. malate/g. wet weight) respectively. Further experiments on the distribution of malate and fumarate in plants will be reported separately. Fumaric acid was found in *G. luteum* by Schmalfuss (1923) and in *Myrrhis odorata* by Itallie & Steenhauer (1926).

*The detection of fumarase activity
at pH 5.0*

Materials were tested for fumarase activity at pH 5.0 as follows: up to 3 ml. of the material to be examined, brought to pH 5 with hydrochloric acid or sodium hydroxide, were placed in the main compartment of vessels containing, in addition, 0.1 ml. buffer-co-factor solution and 0.2 ml. 0.1 M-fumarate in the main compartment and 0.2 ml. cell suspension and 0.1 ml. buffer-co-factor solution in the side arm. Fumarase activity in the test system is shown by a steady carbon dioxide evolution on mixing (above the blank which contains no fumarate). In this way the presence of fumarase has been demonstrated in homogenates of mammalian and avian tissues. A suspension of ground *Gastrophilus intestinalis* larvae showed high fumarase activity; no fumarase activity was detected in extracts of leaves of *Bryophyllum calycinum*, *Sedum praealtum* and *Glaucium flavum*, in rhubarb petiole, *Helianthus tuberosus* tubers, or stored suspensions of intact cells of *Escherichia coli* and *Clostridium welchii*. These negative results do not prove the absence of fumarase from the materials examined, since pH 5 is well below the pH optimum of the enzyme.

The fumarase equilibrium. The method was used to measure the equilibrium ratio malate/fumarate at pH 5.0. At 30° a steady ratio was attained within an hour when 2.5 g. wet weight of frozen yeast was added to 200 μ mol. fumarate. At 25°, 5 g. of yeast gave a constant ratio after 6 hr. A mean value of malate/fumarate at 25° of 10.1 (from seven experiments) was found by measuring separately malate and malate plus fumarate.

Results obtained at pH 7.4 agreed with the mean value of 3.54 reported earlier by Krebs *et al.* (1940). At 30°, equilibrium ratios (malate/fumarate) of 3.41 and 3.46 were obtained, again by separate measurement of malate only and malate plus fumarate.

DISCUSSION

The manometric method is more accurate, specific and sensitive than the polarimetric malate estimation (see Krebs & Eggleston, 1943, 1945). Amounts of L-malate from 1 μ mol. upwards may be estimated, and the accuracy for amounts exceeding 5 μ mol. is within $\pm 1\%$ with special precautions. In comparison with the fluorimetric method of Hummel (1948) and the subsequent modification of Marshall *et al.* (1951) which involves chromatographic separation of the malic acid, the manometric procedure is more specific, rapid and convenient. The malic decarboxylase method for measuring fumarate has advantages over the reduction-ether extraction technique of Krebs *et al.* (1940) in that it is more rapid and independent of the amount of succinate

present. However, the decarboxylase method is inaccurate when the solution contains more than ten times as much malate as fumarate.

The main advantage of the method here described over Ochoa's procedure is the separate measurement of malate and fumarate. Both Blanchard *et al.* (1950) and Ochoa *et al.* (1950) report measurements of the sum of malate and fumarate only in mixed solutions. An additional advantage is the use of the cell suspensions made possible by the preserving action of potassium chloride. Suspensions and acetone powder extracts have similar specificities, and suspensions retain their activity well in 0.1 M-potassium chloride; therefore the preparation of acetone powder extracts, which involves a considerable loss of enzyme, is unnecessary; lastly, the use of bacterial suspensions makes unnecessary the addition of cozymase (required when acetone powder extracts are used for the estimation).

SUMMARY

1. The decarboxylase method for determining the sum of L-malate and fumarate (Ochoa *et al.* 1950; Blanchard *et al.* 1950) has been modified so that L-malate and fumarate can be measured separately.
2. Malic acid is measured manometrically using washed suspensions of malate-adapted cells of *Lactobacillus arabinosus* 17-5 at 35° in a solution of pH 5.0 containing 2.5×10^{-3} M-glucose, 2.5×10^{-3} M-manganous chloride and 10^{-1} M-potassium chloride.
3. Maximum yields of the enzyme are obtained by growing the organism for 24 hr. at 30° on media containing 10^{-1} M-DL-malate, with a second addition of 0.1 vol. M-DL-malate 12 hr. after inoculation.
4. Oxaloacetate, pyruvate and fumarate also form carbon dioxide. Oxaloacetate may be removed with aniline, pyruvate fixed with hydroxylamine or semicarbazide and fumarate removed by reduction with zinc and phosphoric acid, so that malate may be measured accurately in samples containing these acids.
5. Washed cells retain most of their malic decarboxylase activity for several weeks at 2° when suspended in 0.1 M-potassium chloride. Inactive cells may be partly reactivated by addition of cozymase or incubation with glucose and nicotinic acid.
6. Of some forty substances tested only *n*-hexadecyltrimethylammonium bromide (cetavlon), iodoacetate, tungstate, trichloroacetate, triethylamine, 2:4-dinitrophenol, copper sulphate, aluminium sulphate and ferrous sulphate inhibit sufficiently at 10^{-2} M to interfere with the estimation; at 10^{-4} M only cetavlon inhibits severely.
7. The sum of fumarate and malate is measured by adding an excess of fumarase, and the fumarate content is obtained by difference.

8. Fumarase is progressively inactivated by suspensions of *Lactobacillus arabinosus* at pH 5.0 and 30°.

9. Animal tissues were found to contain between 1 and 13 $\mu\text{mol./g.}$ wet weight malate plus fumarate. All plant materials tested contained malate (between 10 and 200 $\mu\text{mol./g.}$ wet weight), whilst fumarate was absent from most materials. The occurrence of fumarate in *Myrrhis odorata* and *Glaucium flavum* (13 and 99 $\mu\text{mol./g.}$ wet weight respectively) has been confirmed.

10. The haemolymph of *Gastrophilus intestinalis* larvae contained exceptionally high concentrations of malate and fumarate (24–38 $\mu\text{mol./ml.}$); these acids account largely for the anion deficit reported by Levenbook (1950).

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The Composition of the Bulk Proteins of *Chlorella*

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Little information is at present available regarding the nature of the algal proteins. Mazur & Clark (1938, 1942) have given the amino-acid composition of the proteins of several species of marine algae, and one fresh-water alga. However, Lugg (1949) has recently questioned the accuracy of the values obtained, and doubted the possible phylogenetic implications. Hot formic acid was used to extract the proteins from the defatted algae, and since this procedure was only 70–80 % efficient, the resulting protein fractions may not have been representative of the whole. Analyses of the acid hydrolysates of the extracted material accounted for 76–88 % of the total protein nitrogen, though of this nitrogen as much as 20 % was combined in the form of humin. No other complete analyses of algal proteins appear to have been published.

This paper presents the results of an investigation of the amino-acid composition of the bulk protein of the unicellular alga, *Chlorella vulgaris*, and is a continuation of the qualitative work reported earlier (Fowden, 1951b). The analyses were made on

a protein fraction believed to be representative of the whole, using the technique of paper chromatography.

EXPERIMENTAL

Material. *Chlorella vulgaris* was grown on the medium of Pearsall & Loose (1936) in penicillin culture flasks maintained at 25° under continuous illumination; the flasks were occasionally gently shaken. When nearing the end of its phase of exponential growth (6–7 days), the *Chlorella* was harvested by centrifugation at 500 g, and then twice washed with distilled water.

Protein extraction. The method used was based on the borate-ether-ethanol method of Lugg (1939). The freshly harvested *Chlorella* cells (about 2 g. on a dry-wt. basis) were suspended in 35 ml. of sodium borate solution (1.1 g./l.), and then the cell walls were broken, releasing the cytoplasmic proteins, by applying a shearing stress of 20,000 lb./sq.in. (Milner, Lawrence & French, 1950). Haemocytometer counts showed that 8–10 % of the cells remained unbroken after this treatment. A sample of the suspension was taken for total-nitrogen assay. The bulk of the suspension was diluted to 250 ml. with more sodium borate solution and 250 ml. of an ether-ethanol mixture (1 vol. ether to 4 vol.